

# High chromosomal variation in wild horn fly *Haematobia irritans* (Linnaeus) (Diptera, Muscidae) populations

Natalia S. Forneris<sup>1</sup>, Gabriel Otero<sup>1</sup>, Ana Pereyra<sup>2</sup>, Gustavo Repetto<sup>1</sup>,  
Alejandro Rabossi<sup>3</sup>, Luis A. Quesada-Allué<sup>3</sup>, Alicia L. Basso<sup>1</sup>

**1** Cátedra de Genética, Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453, C1417DSE CABA, Argentina **2** Departamento de Producción Animal, Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453, C1417DSE CABA, Argentina **3** IIBBA-CONICET, Fundación Instituto Leloir and Dept. Biological Chemistry, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Patricias Argentinas 435, C1405BWE CABA, Argentina

Corresponding author: Alicia L. Basso ([abasso@agro.uba.ar](mailto:abasso@agro.uba.ar))

Academic editor: V. Gokhman | Received 1 September 2014 | Accepted 4 December 2014 | Published 5 February 2015

<http://zoobank.org/9E9D5E84-2782-4D37-9909-960BCDD0A118>

**Citation:** Forneris NS, Otero G, Pereyra A, Repetto G, Rabossi A, Quesada-Allué LA, Basso AL (2015) High chromosomal variation in wild horn fly *Haematobia irritans* (Linnaeus) (Diptera, Muscidae) populations. Comparative Cytogenetics 9(1): 31–50. doi: 10.3897/CompCytogen.v9i1.8535

## Abstract

The horn fly, *Haematobia irritans* is an obligate haematophagous cosmopolitan insect pest. The first reports of attacks on livestock by *H. irritans* in Argentina and Uruguay occurred in 1991, and since 1993 it is considered an economically important pest. Knowledge on the genetic characteristics of the horn fly increases our understanding of the phenotypes resistant to insecticides that repeatedly develop in these insects. The karyotype of *H. irritans*, as previously described using flies from an inbred colony, shows a chromosome complement of  $2n=10$  without heterochromosomes (sex chromosomes). In this study, we analyze for the first time the chromosome structure and variation of four wild populations of *H. irritans* recently established in the Southern Cone of South America, collected in Argentina and Uruguay. In these wild type populations, we confirmed and characterized the previously published “standard” karyotype of  $2n=10$  without sex chromosomes; however, surprisingly a supernumerary element, called B-chromosome, was found in about half of mitotic preparations. The existence of statistically significant karyotypic diversity was demonstrated through the application of orcein staining, C-banding and H-banding. This study represents the first discovery and characterization of horn fly karyotypes with  $2n=11$  ( $2n=10+B$ ). All spermatocytes analyzed showed 5 chromosome bivalents, and therefore,  $2n=10$  without an extra chro-



mosome. Study of mitotic divisions showed that some chromosomal rearrangements affecting karyotype structure are maintained as polymorphisms, and multiple correspondence analyses demonstrated that genetic variation was not associated with geographic distribution. Because it was never observed during male meiosis, we hypothesize that B-chromosome is preferentially transmitted by females and that it might be related to sex determination.

### Keywords

Karyotypes, genetic variability, population structure, B-chromosome, H-banding, evolution, chromosomal rearrangements

## Introduction

The economic importance of the horn fly, *Haematobia irritans* (Linnaeus, 1758) (Diptera: Muscidae) is based on its role as an obligate bloodsucking ectoparasite that plagues cattle around the world (Palmer and Bay 1981, Williams et al. 1985, Valério and Guimarães 1983). First reported in Argentina and Uruguay in 1991 (Luzuriaga et al. 1991, Carballo and Martínez 1991), it has been considered an economically important species in both countries, that can be found from the tropical North (22°02.22'S) to the temperate South (36°18.55'S) and between longitudes 64°33.67'W and 56°54'W (Anziani et al. 1993). Despite its economic importance, no information on the rate of eventual new invasions of this fly is available. In Argentina, the region called “Humid Pampa” has the most favourable conditions for the expansion of this insect and several generations per year, frequently produce sudden, extensive and damaging infestations of cattle (Bulman et al. 1999, Mancebo et al. 2001). Moreover, many local populations from Central and North Argentina demonstrated resistance to fenvalerate and pyrethroids (Torres et al. 1996, Sheppard and Torres 1998, Guglielmone et al. 2001), indicating significant changes in the descendants of the original invading stock that could make them even more invasive and destructive in the future. Insecticide resistance is one of the best examples of rapid microevolution found in nature (Silva et al. 2012) and has strong economical implications. Given the widespread presence of this pest in South Brazil and Paraguay (Sheppard and Torres 1998), and the prevalent cattle trade among these neighbouring countries, the horn fly population in Argentina is likely to be subjected to gene flow.

LaChance (1964) and Avancini and Weinzierl (1994) reported that *H. irritans* showed a karyotype of  $2n=10$  composed of five pairs of chromosomes without a heteromorphic pair of sex chromosomes. As an insect source, all these researchers examined the same laboratory colony maintained at the USDA Livestock Insects Research Laboratory which, in turn, was derived from flies collected in the field at Kerrville, Texas (USA) and reared at least from 1962 on. Apart from *H. irritans*, the only cyclorhaphan Diptera having  $2n=10$  without a heteromorphic pair of chromosomes is *Muscina stabulans* (Fallen, 1817) (Parise et al. 1996, 2007). To our knowledge, no detailed cytological studies of wild horn flies are available.

Here we for the first time analyze wild populations of *H. irritans* which have recently established in the Southern Cone of South America and exhibit considerable



karyotypic diversity. The only related study is the analysis of genotypic variability in three horn fly populations from Brazil, Colombia and Dominican Republic, which was assessed by random amplification of polymorphic DNA (Gatto Brito et al. 2008). In addition, the mitochondrial genome of *H. irritans* was studied by Oliveira et al. (2008).

## Materials and methods

### Ethics statement

This study was carried out on private lands, with kind permission from the landowners. For data regarding permissions for field collecting, please contact the corresponding author. Furthermore, we previously confirmed that no official permission was required for this field work, as it did not involve any endangered or protected species.

### Collection of adult horn flies and eggs

Sampling of adults from different populations of *H. irritans* feeding on cattle was performed from 2004 to 2010 on private lands at the following main stations in Argentina: Ferreyra in Córdoba Province (31°56.67'S; 61°01.0'W); Trancas in Tucumán Province (26°21.66'S; 65°03.0'W); and Bolivar in Buenos Aires Province (35°50.0'S; 64°01.0'W). Sampling of adult *H. irritans* from Uruguay was performed in Palmitas (33°25.0'S; 58°07.0'W). Under normal weather, populations of *H. irritans* develop in these countries from early spring (October) to the beginning of fall (late March) (Guglielmone et al. 2001, 2002, Tarelli 2004), with the average degree of infestation depending on the climate. Samples showing high mortality when transferred from the field to the laboratory were discarded.

Adults were collected on livestock with a sweep net and transferred using positive phototropism to cages with rags soaked with 0.05% sodium citrate-added bovine blood as food source (Filiberti et al. 2009). Females were allowed to oviposit on pieces of cloth impregnated with 8.5 g/l NaCl solution for 12±1 h at 30 °C. After hatching, first-instar larvae were transferred to batches of urine-free bovine feces for feeding. Larval development took place in an environmental chamber at 29±1 °C. The life cycle of *H. irritans* was partially described elsewhere (Basso et al. 2011).

### Cytology

Mitosis and meiosis were studied in neuroblasts of sub-esophageal ganglia of third-instar larvae and in spermatocytes of pharate and freshly eclosed adults respectively. Unfortunately, it was logistically impossible to simultaneously dissect brains and gonads in the same larvae.



### **Preparation of ganglia**

Ganglia were dissected in a drop of insect Ringer's solution (Hayes 1953) and transferred first to 1% sodium citrate solution for 10–15 minutes, and then to methanol/acetic acid (3:1) for 40 seconds. After that, using a slide previously soaked in chilled methanol, each ganglion was transferred into a drop of freshly prepared and chilled 60% glacial acetic acid. The ganglion was then disaggregated with a pair of needles and a micropipette to disperse cells. After that, the slide was placed three times on a hot plate (75 °C) for 3 sec, air-dried, dehydrated, and stored at -20 °C before use.

### **Preparation of testes**

Testes of pharate adult males (120 h after puparium formation) and eclosed adults up to 36 h after emergence were used (Basso et al. 2011). The tissues were fixed and stained in a drop of lacto-acetic 2% orcein solution, covered with a cover slip and squashed 10 minutes later. Preparations with mitotic and meiotic metaphases were then sealed and stored at 6 °C before use.

### **Chromosome banding and idiograms**

Mitotic chromosome spreads from cerebral ganglia were C-banded using  $\text{Ba}(\text{OH})_2$  treatment at 27–29 °C for 7 min and stained with 5% Giemsa Gurr (Merck, Germany) solution in phosphate buffer having pH=6.8 (Basso et al. 1995). H-banding was carried as described by Gatti et al (1976). The cytological preparations, described above, were re-hydrated with phosphate buffer having pH=7 (0.15 M NaCl, 0.03 M KCl and 0.01 M  $\text{Na}_2\text{HPO}_4$ ) for 5 min. The slides were stained with 0.5 µg/ml Hoechst 33258 diluted in phosphate buffer during 10 min and then briefly rinsed with deionized water and air-dried. Mounting was performed in McIlvaine buffer with pH=7 (0.16 M dibasic sodium phosphate, 0.04 M sodium citrate). Preparations were kept in the dark during 24 hours before examination under a Zeiss Axioplan fluorescence microscope. Images were recorded with an Olympus DP72 digital camera, time exposure being manually adjusted. The relative chromosome length and centromere index were calculated after measurements taken both on preparations of ganglia and testes. The idiograms were drawn based on these measurements. Data were obtained from at least 10 metaphase plates per chromosome spread. Circa 1000 insects were dissected to obtain 287 individuals with good quality metaphases.

### **Statistical analysis**

Multiple Correspondence Analysis (MCA) is a method of factorial analysis that transforms a set of categorical or qualitative variables into a small number of orthogonal



variables (Le Roux and Rouanet 2004). We grouped the individuals by seven karyotypic formulae. The variables that characterized the individuals were: the karyotypes classified into seven groups, the chromosome number ( $2n=10$  or  $2n=11$ ), and the presence of satellites on each chromosome of the haploid karyotype (satellite [s1, s2, s3, s5] or absence of satellite). These 14 variables were used as active ones, while the geographical locations, i.e. Bolivar, Palmitas, Trancas and Ferreyra, were included as illustrative variables (Escofier and Pagés 1992, Lebart et al. 1995, 1996). Published data from Texas (LaChance 1964, Avancini and Weinzierl 1994) were also used in the analysis as illustrative individuals ( $n=24$ ), i.e. as unique previous data to make comparisons. With the factorial axes from MCA, a hierarchical classification by Ward was imposed (Johnson and Wichern 1992). Data were processed using SAS software (2009) and SPAD3 (Lebart et al. 1996).

## Results

Chromosomes of four wild horn fly populations from Argentina and Uruguay were studied by cytological analysis of mitotic and meiotic metaphases. Brains of 232 third-instar larvae from three locations (Ferreyra,  $n=134$ ; Bolivar,  $n=25$ ; Palmitas,  $n=73$ ) and testes of 55 individuals from three locations (Ferreyra,  $n=16$ ; Trancas,  $n=14$ ; Palmitas,  $n=25$ ) were analyzed.

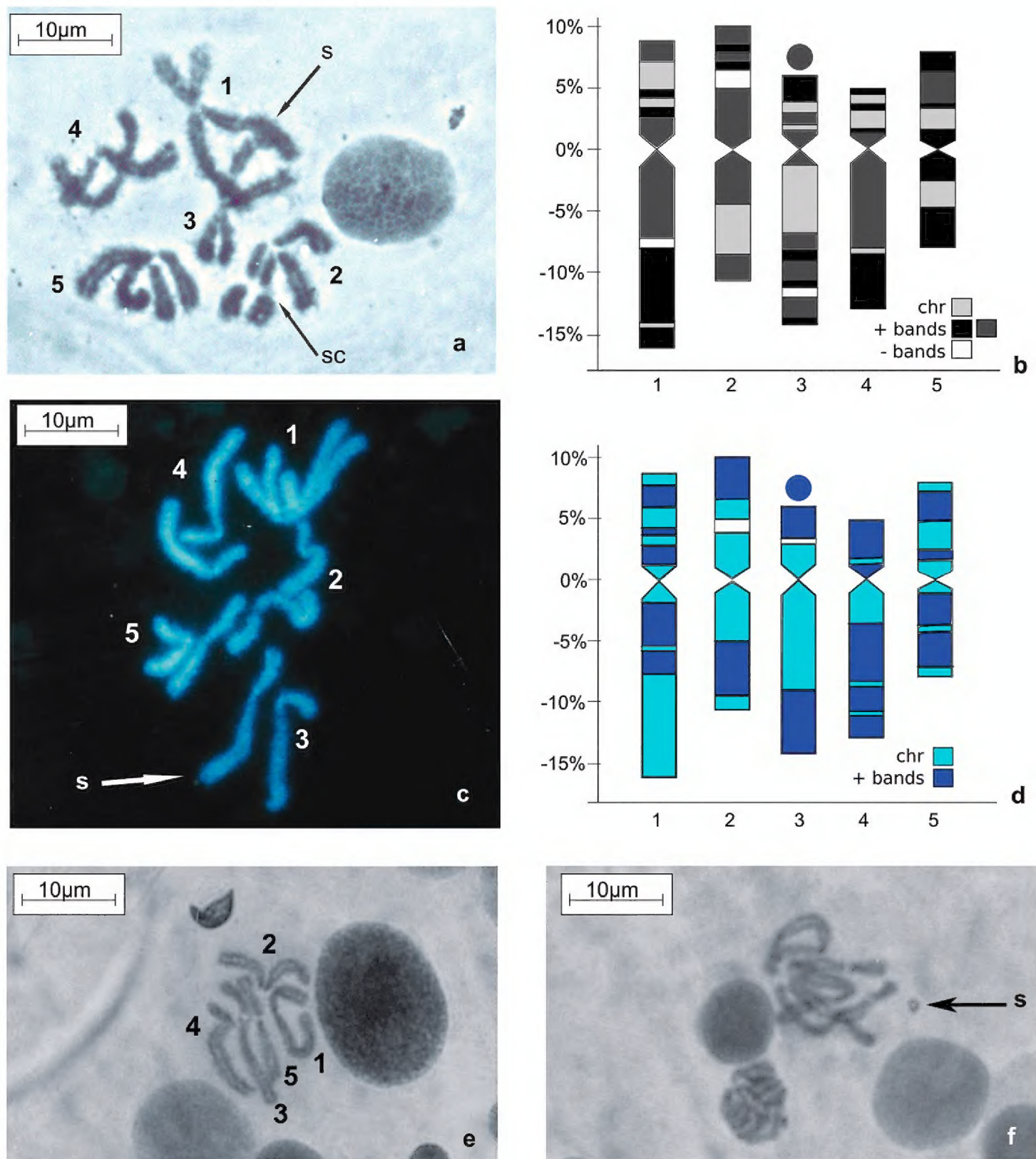
Brain cell preparations from 72 to 96-hour third-instar larvae with empty guts showed mitotic pro-metaphases, metaphases and anaphases. Somatic pairing was observed in metaphase plates as reported for other dipterans (Stevens 1908, Metz 1916, Joyce et al. 2012). We distinguished two types of chromosome numbers within the studied samples:  $2n=10$  and  $2n=11$ . All spermatocytes analyzed showed only 5 chromosome bivalents, i.e.  $2n=10$ . No extra chromosome was found. Preparations from ovaries did not allow for chromosomal analysis since only interphase nuclei at the karyosome stage were present.

### Karyotype I: reference karyotype

Most specimens ( $n=150$ ) were analyzed from Ferreyra. The most frequent chromosome number from this location was  $2n=10$ , both on preparations from brains and testes. We arbitrarily defined the corresponding chromosome set as the reference karyotype (Karyotype I).

Karyotype I showed two metacentric chromosomes (pairs 2 and 5) and three submetacentric chromosomes (pairs 1, 3 and 4) (Fig. 1a–f and Table 1). Chromosome 2 had a distinctive secondary constriction (Fig. 1a–b) which is the location of the nucleolar organizer (not shown), and chromosome 3 carried a satellite (Fig. 1a–b and Table 1). Sex chromosomes were not identified since no heterochromosomes (XY) could be distinguished from the autosomes. In male meiosis, a satellite on chromosome 3 was clearly observed in some spermatocytes (Fig. 1e–f).





**Figure 1.** Reference karyotype of *H. irritans* with  $2n=10$ . **a** Giemsa stained C-banded mitotic metaphase from larval brain; arrow indicates satellite on chromosome 3, sc: secondary constriction **b** Idiogram of the C-banded haploid set **c** Hoechst 33258 stained H-banded mitotic metaphase from larval brain **d** Idiogram of the H-banded set **e–f** Orcein stained meiotic metaphases II from testis; both figures were observed in the same individual. **a, c, f** arrow indicates satellite on chromosome 3. sc: secondary constriction on chromosome 2.

C-banding of karyotype I allowed for detection of satellites (Fig. 1a). The idiogram of C-banded haploid karyotype I is shown on Fig. 1b. Chromosome 1 carried a wide C-block on the long arm and one distal band on the short arm. Chromosome 2 showed a conspicuous C-negative band on one arm that marked a secondary constriction. The long arm of chromosome 3 showed three narrow distal marks and a secondary constric-



**Table 1.** Frequency distribution of karyotypes within populations of *H. irritans* from Argentina and Uruguay. sat: Chromosome carrying satellite; (a) grouping of karyotype formulae in the Multiple Correspondence Analysis.

A. Frequent karyotypes	Ferreyra n=150	Bolivar n=25	Trancas n=14	Palmitas n=98	(a)
Chromosome number 2n=10					
2M + 3SM					
Karyotype I (reference karyotype) 2M (2, 5) + 3SM (1, 3, 4); 3 sat	0.40	0.24	0	0.44	1
Karyotype II 1 sat	0.08	0	0.57	0.01	1
Karyotype III 2, 5 sat	0	0.24	0	0	1
Karyotype IV 3SM (2,3,4); 2 sat	0.02	0	0	0	2
1M + 4SM					
Karyotype V (inversion) 1M (2) + 4SM (1, 3, 4, 5); 1 sat	0.02	0	0.43	0.02	3
Chromosome number 2n = 11					
2M + 3SM + B					
Karyotype VI (translocation 1-5) 2M (1, 2) + 3SM (3, 4, 5) + B; 3 sat	0.32	0	0	0.43	4
1M + 4M/SM + B					
Karyotype VII (inversion) 1M (2) + 4M/SM (1, 3, 4, 5) + B; 2 sat	0.007	0.40	0	0	5
B. Rare karyotypes	Ferreyra n=150	Bolivar n=25	Trancas n=14	Palmitas n=98	
Chromosome number 2n=10					
5SM					
Karyotype VIII 5 SM (1, 2, 3, 4, 5)	0	0	0	0.01	6
1M/SM up to 5M/SM, chromosomal polymorphisms with complex rearrangements					
Karyotype IX (translocation 2-4) 1M (5) + 3SM (1, 3, 4) + 1M/SM (2); 2 sat	0.013	0	0	0	6
Karyotype X (translocation 2-3) 1M (5) + 2SM (1, 4) + 2M/SM (2, 3); 2 sat	0.047	0	0	0	6
Karyotype XI 1M (2) + 2SM (3, 4) + 2M/SM (1, 5); 1, 5 sat	0	0	0	0.01	6
Karyotype XII 1M (2) + 2SM (3, 4) + 2M/SM (1, 5); 2, 5 sat	0.007	0	0	0.01	6
Karyotype XIII (inversion on chromosome 4) 2SM (1, 3) + 3M/SM (2, 4, 5)	0.013	0	0	0.02	6
Karyotype XIV (inversions) 1M (5) + 1SM (2) + 3M/SM (1, 3, 4)	0.007	0	0	0,01	6
Karyotype XV (translocations) 1SM (4) + 4M/SM (1, 2, 3, 5); 2 sat	0.02	0.04	0	0	6
Karyotype XVI 5 M/SM (1, 2, 3, 4, 5)	0.006	0	0	0.01	6
Chromosome number 2n = 11					
Mosaic specimens carrying nuclei with free or attached B chromosomes: formulae with heteromorphic pairs: SM ≠ SM+B					
Karyotype XVII (inversion on chromosome 4) 2M (2, 5) + 2SM (1, 3) + [1SM+B] (4); 2, 3 sat	0.02	0.04	0	0.01	7



Karyotype XVIII (inversion on chromosome 3) 1M(2) + 2SM(1, 4) + 1M/SM (5) + [1SM+B] (3); 2 sat	0.007	0	0	0.01	7
Karyotype XIX (inversions on chromosomes 3, 4) 2M (2, 5) + 1SM(1)+ [2SM+B] (3, 4); 2 sat	0.013	0.04	0	0.01	7
Total	1	1	1	1	

tion followed by a conspicuous satellite (Fig. 1b). Chromosome 4 carried a wide C-block nearly occupying the entire long arm. Chromosome 5 showed a narrow interstitial positive band within a distinct C-banded arm while the other arm revealed no bands. All chromosomes showed pericentromeric C-bands at least in one arm (Fig. 1a–b).

H-banded karyotype I is documented on Fig. 1c. The idiogram of the H-banded haploid set is shown on Fig. 1d. Prominent H-bands were observed in all the chromosomes, and apart from chromosome 4, H-bands did not mark the centromeres. A secondary constriction on chromosome 2 was marked by a gap, i.e. a negative H-band. Chromosome 3 carried positive H-bands on the satellite and the distal regions of both arms. Chromosomes 4 and 5 had large H-bands; chromosome 4 carried a centromeric H-band on the short arm (Fig. 1c–d).

### Chromosomal rearrangements and heteromorphisms

Using karyotype I as the reference one, somatic chromosomal polymorphisms affecting chromosome number and/or morphology were detected. Cytological preparations with  $2n=11$  but without a heteromorphic chromosome pair were selected. This karyotype is formed due to presence of an acrocentric chromosome carrying a small C-positive short arm (Fig. 2a, b, c1, c2, d and Table 1). A summary of variants is represented in the composite C-banding idiogram (Fig. 2b). Chromosome 2 always carried a secondary constriction in all individuals from different populations (see below). Additionally, C- and H-banding revealed variation in the number and position of the bands and in the presence of satellites on chromosomes 1, 2, and 5; no satellite was found on chromosome 4 ( $n=287$ ) (Fig. 2c1, e, g and 3a). Major changes in C-heterochromatin distribution affected pairs 1, 2 and 5 (Fig. 1b and 2b). The polymorphic long arm of chromosome 3 showed a curved shape on one homologue, lack of pairing and a change in the position of secondary constriction (Fig. 2b, e, e2). A variant of chromosome 4 showed change in C-heterochromatin distribution on the long arm: one of the homologs exhibited the darkest band on the long inverted arm, leading to lack of pairing (Fig. 1b, 2b, e). In the same specimen, a chromosomal bridge (2e1) as well as another metaphase plate (Fig. 2e2) with polymorphic pairs 3 and 4, add further evidence for these rearrangements, as they could arise from an inversion. A submetacentric variant of chromosome 5 was revealed by C-banding (Fig. 2a, b and e). The extra small chromosome was positively H-banded (Fig. 2d). Unpaired chromosomal segments were recognized in two metaphases of the same specimen (Fig. 2f1, f2), indicating banding heteromorphisms and structural polymorphisms.



## Frequent and rare karyotypes

We identified 19 main chromosomal profiles in Argentina and Uruguay (Table 1), through differences in the chromosome banding (heteromorphisms), chromosome rearrangements (polymorphisms) and chromosome number (Fig. 2a, c–g and 3a–d); frequency distribution of these profiles within each population was calculated. These profiles were classified in seven chromosomal formulae (Table 1). Table 1A groups the most frequent chromosomal formulae ( $\geq 0.150$ ) with the exception of formula 1M + 4SM (see below), whereas rare karyotypes are shown in Table 1B.

Karyotypes composed of two metacentric and three submetacentric chromosomes (formula 2M + 3SM) are represented by variants I, II, III (Table 1A, column “a” Group 1) and IV (Table 1A, column “a” Group 2). Karyotype II is similar to karyotype I but with a satellite on chromosome 1. Karyotype III showed satellites on chromosomes 2 and 5. Karyotype IV has undergone a significant change that gave rise to metacentric chromosome 1 and submetacentric chromosome 2 (Fig. 3b1 and b2) and thus we used it in our Multiple Correspondence Analysis (Table 1, column “a” Group 2). Karyotype V has one metacentric (chromosome 2) and four submetacentric chromosomes, i.e. 1M + 4SM (Table 1, Group 3); chromosome 1 carries a satellite (Fig. 2g). This karyotype is dominant in Trancas population.

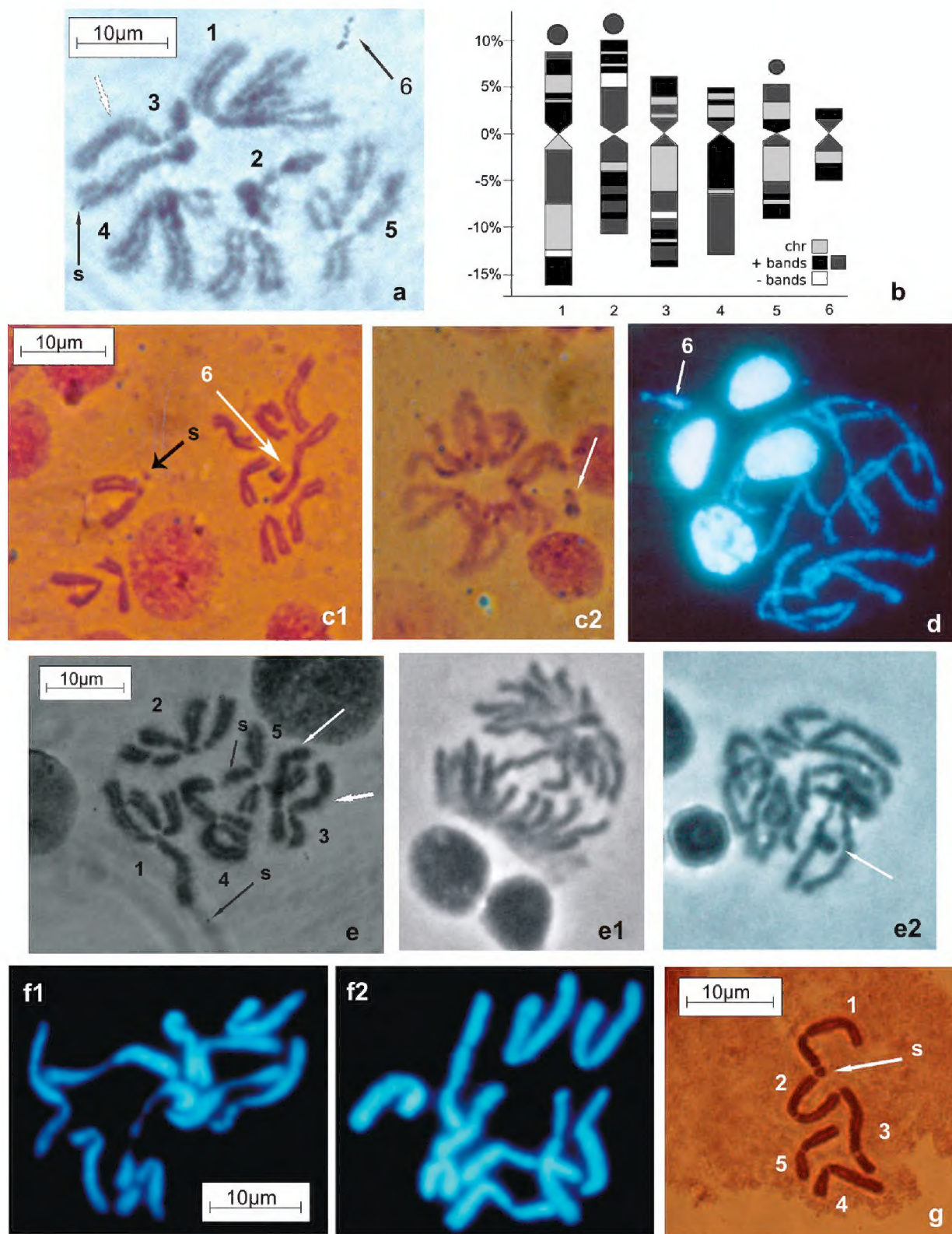
Karyotypes with  $2n=11$  ( $2n=10+B$ ) and formula 2M + 3SM + B correspond to karyotype VI (Fig. 2a) (Table 1A, Group 4), whereas those with formula 1M + 4M/SM + B carrying four polymorphic pairs formed by one metacentric and one submetacentric chromosome correspond to karyotype VII (Fig. 2c1 and c2) (Table 1A, column “a” Group 5). Karyotype VI appeared to be dominant in our samples from Ferreyra and Palmitas, whereas karyotype VII was dominant in Bolivar.

Rare karyotypes (Table 1B) were not found in the Trancas population. Formula 5SM corresponds to karyotype VIII ( $2n=10$ ), composed of five pairs of submetacentric chromosomes (not shown). Only one specimen of that kind was found in the Palmitas population (Table 1B column “a” Group 6). The sixth formula, 1M/SM to 5M/SM ( $2n=10$ ) includes eight very scarce karyotypes (IX to XVI) from Ferreyra and Palmitas, carrying 1 to 5 polymorphic pairs composed of one metacentric and one submetacentric chromosome (Table 1B, Group 6). C- and H- bandings provide strong evidence for complex rearrangements along with lack of somatic chromosomal pairing (Fig. 2d, 2f1, 2f2, 3b1, 3b2, 3c1, 3c2). Rare karyotypes XVII, XVIII and XIX with formula  $SM \neq SM + B$  (Table 1B, column “a” Group 7) include mosaic insects carrying nuclei with  $2n=10$  and  $2n=11$  and having polymorphic pairs of chromosomes (Fig. 3a); the extra chromosome is free or attached to one of the homologs of pair 3 or pair 4 (Table 1B, Group 7) (Fig. 2e, e1, e2).

## Population structure

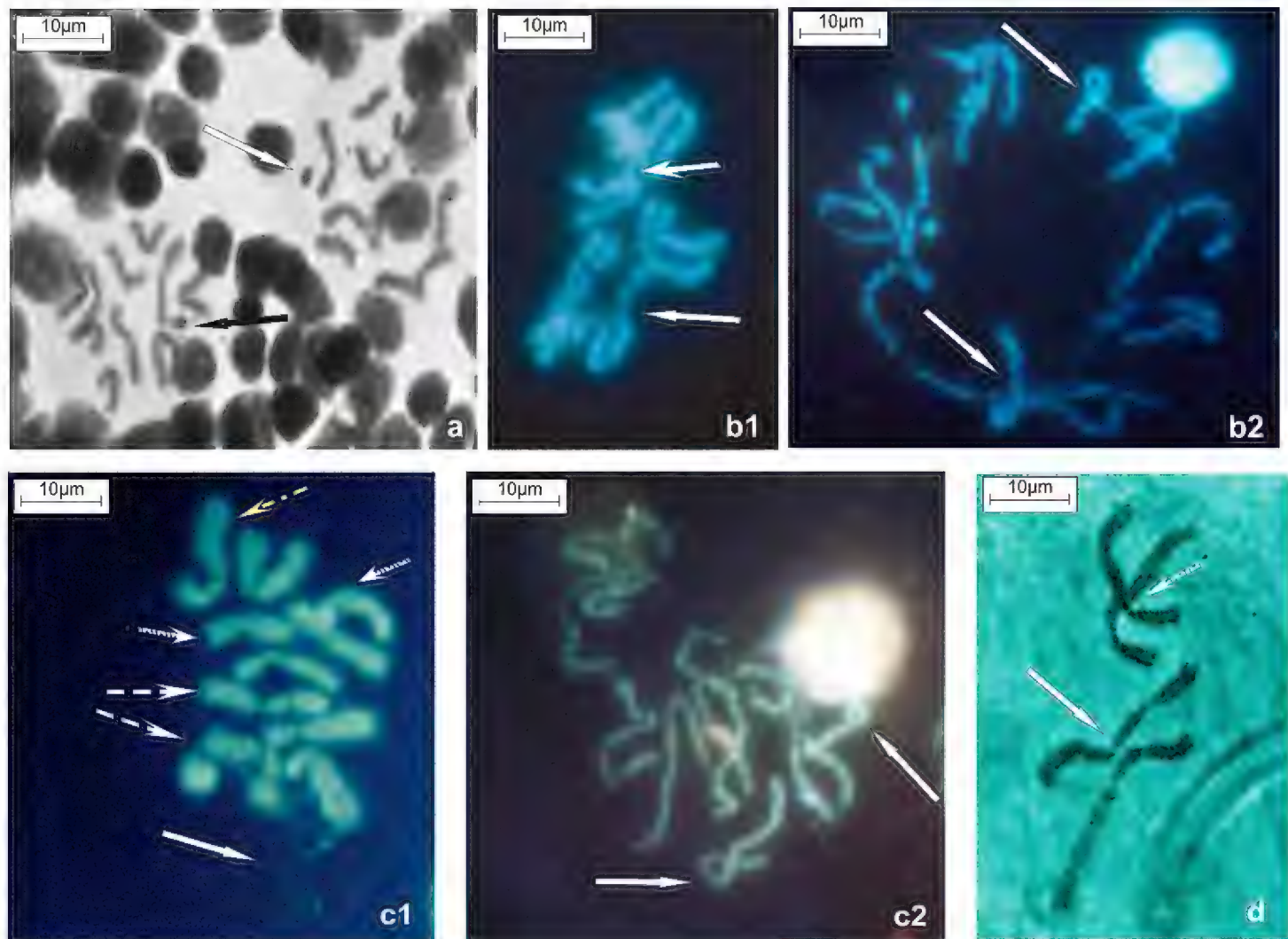
Within the Ferreyra population, karyotype I ( $2n=10$ ) and karyotype VI ( $2n=11$ ) were present in 40% and 32% of the insects respectively (Table 1). Due to the fact that the





**Figure 2.** Chromosome variants in *H. irritans*. **a–f** mitotic plates from larval brain cells. **a** Giemsa C-banded metaphase plate with  $2n=11$  and karyotype VI; black arrow indicates B-chromosome, white arrow indicates relocation of secondary constriction on the curved long arm of one homologue of pair 3, “s” indicates satellite on chromosome 3 **b** Composite C-banding idiogram showing some chromosome variants found in different specimens **c** Giemsa C-banded metaphases with  $2n=11$  found in the same larva with karyotype VII; white arrow indicates B-chromosome, black arrow indicates satellite on metacentric chromosome 2 **d** Hoechst 33258 stained prometaphase with  $2n=11$  carrying complex rearrangements; white arrow indicates B-chromosome **e** Giemsa stained C-banded metaphase with  $2n=10$  and heteromorphic pairs 3 and 4; black arrows indicate satellites on chromosomes 1 and 5, white arrows indicate both long arms of pair 3 with one of them carrying an attached B-chromosome **e1** anaphase with a bridge **e2** metaphase from the same specimen as in **e** and **e1**; white arrow indicates rearrangement in one of the homologues of pair 3, black arrow on pair 4 **f** Hoechst 33258 stained metaphases with  $2n=10$  and karyotype XV carrying complex rearrangements **g** Orcein stained meiotic metaphase II from testis with karyotype V; white arrow indicates satellite on chromosome 1.



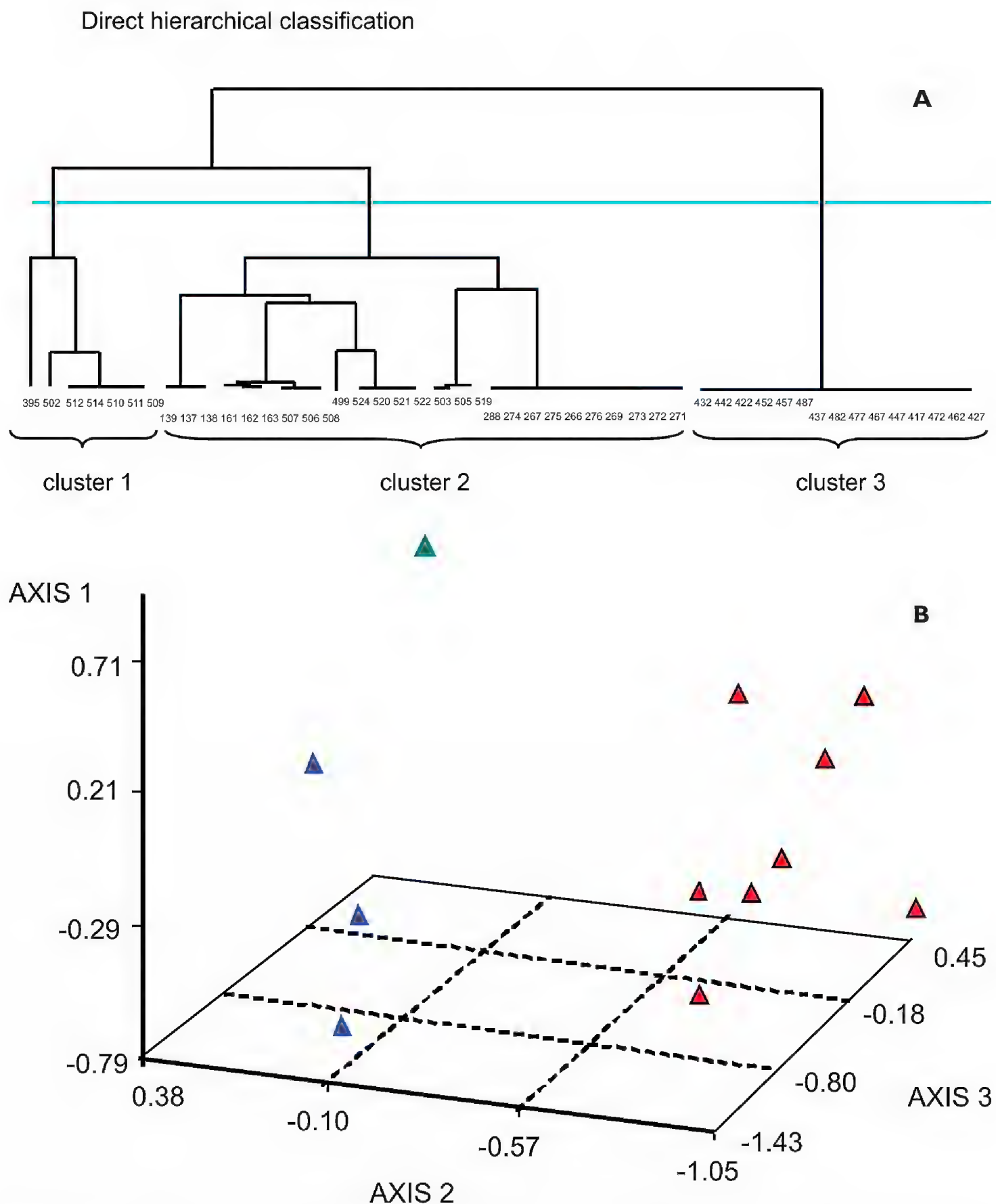


**Figure 3.** Chromosome variants in *H. irritans*. Mitotic plates from larval brain cells. **a** Two C-banded metaphases with karyotype XVIII, one with  $2n=11$  ( $2n=10+B$ ) and the other with  $2n=10$  with free and attached B-chromosomes (white and black arrows respectively) **b1** and **b2** Hoechst 33258 stained metaphase and prometaphase of the same specimen with karyotype IV; arrows indicate rearranged pairs 1 and 2 **c** Hoechst 33258 stained metaphase and prometaphase **c1** heteromorphisms of pairs 1 (dashed yellow arrow), 4 and 5 (dotted arrows) and the nucleolus close to chromosome 2 (dashed white arrow), expression of ribosomal DNA located on secondary constriction on chromosome 2 (white arrow) **c2** H-banded karyotype with heteromorphisms; arrows indicate rearrangements on prometaphase chromosomes **d** C-banded incomplete metaphase with chromosomal pairs 1 and 3 (dotted arrow); white arrow indicates pair 1 carrying heterozygous inversion.

sample size of this population was large ( $n=150$ ), we detected specimens with 16 different karyotypes (Table 1). Insects with  $2n=10$  and chromosome variations represented 23.3% within this population; those included karyotypes II, IV (Fig. 3b1, b2 and Table 1) and all the series of karyotypes from 1M/SM up to 5M/SM (group 6). All the karyotypes with  $2n=11$  (Table 1, groups 4, 5 and 7) were present in 36.7% of Ferreyra specimens. However, the extra chromosome was never observed in preparations from the testes.

Within the Bolivar population, 52% flies had  $2n=10$ . The frequency of karyotype I was 24%. Interestingly, karyotype III with the same frequency and satellites on chromosome 2 was unique to this population (Table 1). Additionally, karyotype VII with  $2n=11$  (Fig. 2a, c1, c2, d, 3a and Table 1) was present in 40% of the insects within Bolivar sample.





**Figure 4.** Clustering and spatial distribution of *H. irritans*. **a** The most significant discrimination is obtained by the first three axes: karyotypes, satellites and zygotic number of chromosomes, and therefore the three clusters are the most representative. Texas individuals (LaChance 1964, Avancini and Wienzierl 1994) were also included **b** Spatial distribution of individuals; clusters 1 to 3 and Texas individuals are shown in blue, red, green and black respectively.

In the Trancas population, only a small sample was studied on the basis of male meiosis. All the insects had karyotype II (2M + 3SM) or karyotype V (1M + 4SM) (Fig. 2g and Table 1). Both karyotypes showed a satellite on chromosome 1, but they



differed in the morphology of chromosome 5, which appeared to be metacentric in karyotype II but submetacentric in karyotype V.

In the Palmitas population, karyotypes I ( $2n=10$ ) (44%) and VI ( $2n=11$ ) (43%) were dominant. Although 56% insects carried rearrangements, flies having  $2n=11$  prevailed (46%; Table 1). Karyotypes II, V, VIII, IX, X, XII and XIV also had structural rearrangements (10%). This was the only population where an individual carrying karyotype VIII with five submetacentric pairs (formula 5SM) was found. Karyotype II was uncommon in Palmitas but frequent in Trancas (Table 1).

MCA produced 14 axes from 14 nominal variables (see Materials and Methods). From these, we have chosen seven factors that explained 92.25% of the whole variation. Graphical displays constructed from cluster analysis obtained from those factors, were used to summarize proximities between the specimens and to show associations between the categorical variables. The dendrogram (Fig. 4a) showed the hierarchical classification, where the greatest discrimination was obtained by the first three factors: karyotypes, satellites and zygotic chromosome number. Therefore the three clusters that grouped similar individuals were the most representative. As expected, the factorial analysis has clearly shown that localities were not a discriminating factor.

Cluster 1 ( $n=141$ ) is characterized by individuals from groups 1 and 3 (Table 1A, column "a"), with  $2n=10$ , and satellites on chromosome 1 (s1), or on chromosome 2, 3 or 5. This cluster grouped all Trancas individuals, 50% of Ferreyra individuals and 47% of Palmitas individuals. Neither specimens from groups 2, 4, 5, 6 and 7 nor those with  $2n=11$  were found in this cluster (Table 1A, column "a").

Cluster 2 ( $n=56$ ) is characterized by all individuals of groups 2, 5, 6 and 7 (Table 1, column "a"); those with  $2n=10$  predominated. All insects with satellites on chromosomes 2 or 5 as well as those without satellites were found in this cluster. There were very few individuals of group 1 (10.71%) and no individuals of group 4 (Table 1, column "a"). Cluster 2 grouped 76%, 10% and 18% of Bolivar, Palmitas and Ferreyra individuals respectively.

Cluster 3 ( $n=90$ ) is characterized by individuals with  $2n=11$ , of group 4 and with a satellite on chromosome 3; it is composed by 32% of Ferreyra individuals and 43% of Palmitas individuals (Table 1A, column "a").

The specimens were represented in three-dimensional graphical displays constructed using the first three factorial axes (Fig. 4b). Data on the flies from Texas (LaChance 1964, Avancini and Weinzierl 1994) are also given (in black); the spatial distribution of all individuals (Fig. 4b) showed them as a part of cluster 2 (in red). The clusters well differ from each other; all individuals in cluster 3 have identical karyotypes.

## Discussion

This study is the first chromosomal analysis of recently established wild populations of the horn fly, originally introduced in Brazil in 1983, in the Southern Cone of South



America. We believe that the karyotype study provides a basic tool for understanding the population dynamics. Unfortunately, this research is limited by the lack of a full-cycle laboratory rearing technique of the horn fly.

### Chromosomal rearrangements

We here document chromosomal rearrangements that affect the chromosome number and morphology. We also confirm that the main chromosome number in *H. irritans* is  $2n=2x=10$ . For the first time we show the existence of an extra chromosome in three populations, resulting in the presence of chromosome sets with  $2n=11$  ( $2n=10+B$ ).

Our study showed that the main chromosomal formula for the karyotypes found in Ferreyra, Palmitas, Trancas and Bolivar was  $2M + 3SM$ . Variation found in the populations from Ferreyra, Palmitas and Bolivar (Table 1), involved some complex chromosomal rearrangements such as those in karyotypes VIII to XVI (Table 1B) and mosaic specimens such as those with karyotypes XVII to XIX carrying nuclei with 10 and 11 chromosomes. Within the best-analyzed populations, i.e. Ferreyra and Palmitas, individuals with  $5M/SM$  or  $5SM$  were found within all clusters (Fig. 4a, b). This karyotype diversity was previously reported neither by LaChance (1964) nor by Avancini and Weinzierl (1994). LaChance (1964) studied chromosome sets of the horn fly on the preparations from larval brain cells ( $n=18$ ) or testes ( $n=11$ ) and described a unique karyotype of *H. irritans* with  $2n=10$  composed by five pairs of autosomes ( $4M + 1 SM$ ) without a distinct secondary constriction. Thirty years later, Avancini and Weinzierl (1994) described individuals from the same colony, but with only one karyotype  $2n=10$ , composed by five pairs of autosomes ( $3M + 2 SM$ ) based on the analysis of “ten adult testis cells in meiotic metaphase” or “a few larval brain cells”; those authors found that submetacentric chromosome 3 seemed to carry the nucleolar organizer in the pericentromeric region. However, we found that chromosome 2 carried a secondary constriction that denotes the location of the nucleolar organizer (not shown) and can reveal a satellite during the cell cycle. Mobility of the satellites between different chromosomes can be explained either by transposition or fragmentation of the nucleolar organizer.

### Origin and transmission of the small extra chromosome

Extra chromosomes were found within karyotypes from Ferreyra, Bolivar and Palmitas, but we could not determine whether it was the same B-chromosome in all cases. A B-chromosome might be derived from a fragmented autosome or a sex chromosome, i.e. a centric fragment derived from amplification of the paracentromeric region (Fig. 2b–e, 3a, Table 1). It could originate by inversions that require at least two breaks on the same chromosome. The small chromosome could arise by a pericentric inver-



sion taking place close to one inessential chromosomal end (3b1, 3b2, 3d). When two crossing-overs involving three chromatids take place, i.e. one within the inverted region and the other within the proximal region, a bridge gives origin to small chromosomes (Fig. 2b, e, e1, e2).

Lampe et al. (1998, 2001) reported the presence of transposons in *H. irritans*, and *HIMAR1* is one of the only two known active mariner elements (Lampe et al. 1998). Heterochromatic B-chromosomes, usually revealed by C-banding, typically originate from satellite blocks of repeated DNA sequences which vary in type, kind of repeat, and copy number (Franks et al. 1996), as a result of unequal crossing-over and reduced recombination (Bigot et al. 1990, Charlesworth and Sniegowski 1994). Camacho et al. (2000) proposed that B-chromosomes might be amalgamations of transposable DNA. As indicated in Table 1, rearrangements affecting chromosomes 3 or 4 were associated with B-chromosomes (Fig. 2e, e2; autosome 3, Fig. 3a). Analyses of C-banding (Fig. 1b, 2a, b, c1, c2, e, e1, e2) and H-banding patterns in brain larval cells (Fig. 1c, d, 2d–f1, f2) at the population level, suggested that the extra small chromosomes might originate through breaks within chromosome 3 or chromosome 4.

Population frequencies of B-chromosomes result from a balance between their transmission rates and their effects on host fitness (Basso and Lifschitz 1995, Camacho et al. 2000). Our observations suggest that a free B-chromosome is only transmitted via female meiosis. Differential transmission of the B-chromosome was cytogenetically studied in *Ceratitis capitata* (Wiedemann, 1824) (Basso and Lifschitz 1995), where 63 progenies from reciprocal crossings were studied and the B-chromosome observed in both sexes, in somatic cells (cerebral ganglia tissue) as a free chromosome, or terminally attached to the long arm of the X-chromosome, appearing as a larger X. Males transmit both free Bs and large X-chromosomes to their progeny. Females of *C. capitata* transmit the large Xs to their progeny, at a higher rate than the standard Xs, suggesting either differential fate of the oocytes or a preferential co-orientation during the first meiotic division. A similar phenomenon that involved preferential survival of spermatozoa bearing Bs was also described in this species by Manso and Lifschitz (1986).

In our studies, the extra chromosomes might be associated with sex determination as the frequencies of individuals with  $2n=11$  in Ferreyra, Bolivar and Palmitas were 0.367, 0.48 and 0.46 respectively. The extra chromosome was observed in around 50% of larval brains and was not observed in preparations from testes, further suggesting that the B-chromosome is restricted to females. We hypothesize that this B-chromosome is preferentially transmitted by females, and that it might be related to sex determination because it was never observed during male meiosis. Sex chromosomes were previously proposed as ancestors of the Bs (Hewitt 1973). At the parsimonious hypothesis, homomorphic chromosomes may have sex determination factor like in *Aedes aegypti* (Hall et al. 2014). In spite of the difficulties to demonstrate B-chromosomes in gonad tissues, we believe that extra chromosomes behave in a similar way that those reported in *C. capitata* (Basso and Lifschitz 1995), because they are transmitted from parents to the offspring.



## Rearrangements, hybridization and polymorphisms

Although a direct investigation of demographic structure is difficult in natural conditions (Roderick 1996), study of the genetic structure of wild horn fly populations that is affected by mutations, migration or gene flow, selection and genetic drift, is a basic tool to understand population dynamics and insecticide resistance that must be complemented by DNA sequencing studies.

MCA generated three clusters: cluster 1 grouped specimens with  $2n=10$  from all four populations; cluster 2 grouped rare karyotypes with  $2n=10$  and  $2n=11$  as well as insects from Texas (Fig. 4a–b); cluster 3 grouped insects with  $2n=11$  from Ferreyra and Palmitas. Since karyotypes from Texas were respectively reported 50 and 20 years ago (LaChance 1964, Avancini and Weinzierl 1994), loss of chromosome variants was probably due to inbreeding, thus both karyotypes were grouped in cluster 2. We did not find the karyotypes from Kerrville within our populations. However, other less frequent chromosome sets carrying heteromorphic M/SM pairs, grouped in the same cluster together with Texas karyotypes. Moreover, it is significant that the correspondence analysis revealed that localities did not discriminate between populations, apart from the chromosome number and karyotype structure (Fig. 4a–b). Since up to 1200 generations of the horn fly during the last 50 years have occurred, metacentric chromosomes that were reported by LaChance (1964) and Avancini and Weinzierl (1994), may have been transformed into submetacentric ones. Inversions which reduced interchromosomal recombination and also maintained the polymorphism, are likely to have been responsible for this significant change. On the other hand, hybridization produced further recombination between the different variants.

Abundant karyotypes were analyzed in two locations, Ferreyra in Argentina and Palmitas in Uruguay. Resilience of genomes to massive introgression through hybridization, can allow for rapid adaptive response to anthropogenic selection (Clarkson et al. 2014). Thus, our results explain repeated appearance of resistant phenotypes in *H. irritans*.

## Conclusions

We confirmed the chromosome set with  $2n=10$  as the reference karyotype in wild populations of *H. irritans* invading the Southern Cone of South America. Karyotypic variants were characterized for the first time; half of these variants were  $2n=11$  due to the presence of a B-chromosome.

The B-chromosome was observed only in mitotic divisions, mainly as a free acrocentric chromosome.

Horn fly control will highly benefit from genetic studies focusing on the understanding of sex determining mechanisms, which are necessary to design appropriate control strategies, as related to the adaptation of these insects to control measures such as insecticides.



## Acknowledgments

We thank Dr. C. Argaraña and Dr. A. Filiberti for providing the Ferreyra specimens. Funding for this project was provided by the ANPCYT-PICT 2003-351, CONICET (Argentina), and the University of Buenos Aires (UBA).

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